Defective synthesis of envelope proteins by temperature-sensitive mutants representing complementation groups B and D of respiratory syncytial virus

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The phenotypes of two complementing temperature-sensitive (ts) mutants of respiratory syncytial (RS) virus indicate that the mutational lesions involve the attachment (G) and matrix (M) proteins of the viral envelope. Synthesis of the G protein was affected in cells infected with mutant tsA2 (complementation group B); the p50 precursor of the G protein was synthesized normally, but further maturation to the fully glycosylated form was defective at 39 °C. A non-ts alteration in the efficiency of cleavage of the Fo precursor to the F1 and F2 subunits of the fusion protein was also observed in tsA2-infected cells, which is consistent with the aberrant non-syncytial plaque morphology induced by tsA2 in certain cells. In cells infected with mutant tsN1 (complementation group D) the M protein disappeared from the soluble cytoplasmic fraction soon after synthesis at 39 °C and had a slightly decreased electrophoretic mobility. The M protein of non-ts revertants was stable at 39 °C, which links the defect in M protein stability with the tsN1 phenotype. However, the aberrant mobility phenotype remained, suggesting pseudoreversion. These results assign two of the eight complementation groups of ts mutants of RS virus.

Introduction

The function of viral proteins can be determined by phenotypic characterization of conditional lethal mutants assigned to different complementation groups.

Previously, induced temperature-sensitive (ts) mutants of the A2 (Gharpure et al., 1969), RSN-2 (Faulkner et al., 1976) and RSS-2 (McKay et al., 1988) strains of human respiratory syncytial (RS) virus have been isolated from wild-type viruses propagated in secondary bovine kidney, BS-C-1 and MRC-5 cells respectively. Mutants of the A2 and RSN-2 strains were assigned initially to seven (Gimenez & Pringle, 1978), later amended to eight (Pringle et al., 1981), complementation groups. Mutants of both strains are represented in complementation group A, whereas groups B and C are unique to the A2 strain, and groups B', D, E, F and G are unique to the RSN-2 strain. On the assumption that these complementation groups correspond to eight of the 10 genes of the human RS virus genome, two groups remain to be identified. Three of the eight groups can now be equated with genes. Evidence from the analysis of phenotypes presented here suggest that complementation groups B and D represent mutational lesions in the attachment (G) and matrix (M) protein genes respectively. Phenotypic and genotypic analyses indicate that complementation group E corresponds to the P protein gene (C. Caravokyri & C. R. Pringle, unpublished results).

Methods

Viruses. In this paper mutants derived from the A2 strain have A preceding the mutant number and those from the RSN-2 strain have N before the number. Mutant tsA2 was obtained originally from L. S. Richardson and R. M. Chanock (NIH, Bethesda, Md., U.S.A.), propagated in BS-C-1 cells and recloned prior to these experiments. A non-permissive temperature of 39 °C was used throughout.

Cells. Maintenance of BS-C-1 cells, virus growth and infectivity assays were carried out by standard procedures (Pringle, 1985). Foetal calf serum (FCS) was obtained from Gibco BRL. Virus stocks were prepared by inoculating monolayers of approximately 3 x 10⁶ BS-C-1 cells in small plastic flasks (Nunc) with approximately 1 p.f.u./ml of ts mutant, revertant or wild-type virus. Cells and supernatant fluid were harvested when c.p.e. was advanced. Infectivity assays were carried out under an overlay containing agar at 33 °C and 39 °C in CO₂ incubators.

Radiolabelling. BS-C-1 cell monolayers in plastic Leighton tubes (5.5 cm²) were infected with approximately 1 p.f.u. virus/ml and incubated for 24 to 48 h until the first appearance of c.p.e. The incubation medium was replaced with 6 ml medium lacking methionine and FCS for 1 h prior to radiolabelling with [35S]methionine. Subsequently the infected cultures were incubated for 18 to 20 h in 0.5 ml methionine-free medium containing 30 to 40 μCi/ml [35S]methionine (Amersham); specific activity >800 Ci/mmol.)
For pulse-chase experiments, replicate infected monolayers were incubated in 6 ml maintenance medium containing 2% FCS until c.p.e. was extensive. The maintenance medium was replaced by medium lacking methionine and FCS, and incubation continued for 3 h. This medium was then replaced by 0·3 ml of medium containing 150 µCi/ml L-[35S]methionine prewarmed to the appropriate incubation temperature. The length of the pulse was 10 min, except where indicated. The pulse medium was then removed and residual radiolabel washed off with two changes of ice-cold maintenance medium supplemented with a concentration of t-methionine 200-fold that normally used. One replicate culture remained on ice as the zero time sample for immediate extraction of proteins, whereas the remaining cultures received 0·5 ml of the prewarmed wash medium and were incubated for varying periods at either 33 °C or 39 °C. A CO2 incubator was used for incubation at 33 °C, and a precision water bath for incubation at 39 °C.

[3H]Leucine was used to detect the G protein because this protein has a low methionine content. Leucine-free maintenance medium was used for pre-exposure incubation and washing after pulse-labelling, and maintenance medium supplemented with a concentration of leucine 50-fold that normally used was added during the chase period.

Radiolabelling of viral glycoproteins was achieved by incubating infected cells in 0·5 ml of maintenance medium containing 2% FCS and 100 to 200 µCi/ml [3H]glucosamine (Amersham; specific activity 20 to 40 Ci/mmol) for 18 to 20 h from the time of onset of c.p.e. [3H]Leucine was used to detect the G protein because this protein has a low methionine content. Leucine-free maintenance medium was used for pre-exposure incubation and washing after pulse-labelling, and maintenance medium supplemented with a concentration of leucine 50-fold that normally used was added during the chase period.

Immunoprecipitation. Radiolabelled virus-infected and mock-infected cell monolayers were detached by shaking with sterile glass beads. The cell debris was collected as a pellet by centrifugation for 30 s at 6000 g. The supernatant was retained and the pellet washed three times by resuspension in 1 ml ice-cold PBS/PMSF. The cell pellet was finally resuspended in 100 µl of PBS/PMSF and the cells were lysed by addition of NP40 to a final concentration of 2% (v/v). After 30 min on ice the debris was removed by centrifugation and the supernatant preclarified by incubation with 50 µl of freshly prepared immunoprecipitin (formalin-fixed Staphylococcus aureus A cells; BRL) and 10 µl FCS for 30 min on ice. The supernatant was clarified before centrifugation and reacted with 4 to 10 µl of polyclonal bovine anti-human RS virus serum (kindly provided by E. J. Stott & G. Taylor, AFRC IAH Compton Laboratory, Compton, U.K.), or a specific monoclonal antibody (MAb) (see text), for at least 4 h at 4 °C. The samples were then incubated with 40 to 100 µl immunoprecipitin for 30 min at 4 °C, and the resulting immune complexes pelleted and washed three times with cold buffer (0·5 M-LiCl in 0·1 M-Tris-HCl at pH 8·5). The samples were boiled in SDS-PAGE buffer for 2 to 3 min and clarified before electrophoresis. PAGE, fluorography and autoradiography were carried out by standard procedures (Cash et al., 1977).

Immunoprecipitation of radiolabelled proteins from released virus particles was carried out similarly, except that the released particles were concentrated by precipitation with 7% polyethylene glycol (PEG) 6000 (BDH) at 4 °C for 16 to 24 h prior to lysis. The precipitate was collected by centrifugation and dissociated by resuspension in 50 µl ice-cold RIP/PMSF for 30 min at 4 °C.

In vitro translation. Total cytoplasmic RNA from infected or mock-infected cells (up to 10 µg) was translated in a nuclease-treated rabbit reticulocyte lysate (NE) as described previously (Caravokyri & Pringle, 1991).

Results

Evidence indicating that a G protein defect determines the ts phenotype of mutant tsA2 (complementation group B)

Mutant tsA2 belongs to the group of mutants isolated from the A2 strain (subgroup A) by Gharpure et al. (1969). This mutant was initially classified with 10 mutants of the RSN-2 strain in complementation group B (Gimenez & Pringle, 1978), but subsequently more stringent complementation assays led to reclassification of the 10 RSN-2 mutants in a separate group, B' (Pringle et al., 1981). Mutant tsA2 is now the only representative of complementation group B. Mutant tsA2 differs from other mutants of the A2 and RSN-2 strains in that it produces non-syncytial plaques in Hep-2 and some other cells at permissive temperatures (Wright et al., 1973; Belshé et al., 1977). This phenotype was not observed in BS-C-1 cells, however, because RS virus produces focal rather than syncytial plaques in these cells (Pringle, 1985).

(i) Viral polypeptide synthesis at 33 °C and 39 °C in cells infected with mutant tsA2

Radiolabelling of viral polypeptides in tsA2-infected cells with [35S]methionine (not shown) indicated that synthesis of the major 35S-labelled species (i.e. the F1, N, P and M polypeptides) of tsA2 at 39 °C was not impaired. This observation agrees with the previous finding of Belshé et al. (1977) showing that growth of tsA2 was not restricted at the higher temperature provided that adsorption had occurred at the permissive temperature. Thus, despite the difference in plaque phenotype, the growth characteristics of tsA2 in BS-C-1 and Hep-2 cells appeared to be the same.

Intracellular viral glycoprotein synthesis in tsA2-infected cells at 33 °C and 39 °C was monitored by [3H]glucosamine labelling and immunoprecipitation with polyclonal antiserum (Fig. 1 a and b) or MAbs (Fig. 1 c and data not shown). In cells infected with mutant tsA2 at 39 °C, the intensity of the band representing mature G polypeptide was reduced markedly relative to that obtained at 33 °C (Fig. 1 a and c). However, synthesis of the major G protein precursor species seemed to be no more restricted at 39 °C than that of the F1 and F2 polypeptides. By contrast, analysis of released virus obtained by PEG precipitation from the culture fluid did not reveal a corresponding deficit of mature G protein at 39 °C (Fig. 1 b). However, the diffuseness of the G protein band did not permit discrimination of the soluble and membrane-inserted forms, which differ in $M_r$ by 6K to 9K (Hendricks et al., 1988), and both forms may be present.

Although the G protein precursor bands p45 and p50 of subgroup A viruses such as RSS-2, A2 and Long (Routledge et al., 1986), and intermediate products, were detectable to varying extents depending on the virus strain and stage of the growth cycle at which radiolabelling was initiated, densitometric analysis of fluorograms from these experiments confirmed that similar amounts of the p45 and p50 precursor stages were present in both
Fig. 1. Synthesis of viral glycoproteins in mutant tsA2-infected cells. (a) Immunoprecipitated lysates of [3H]glucosamine-labelled cells incubated at 33 °C (lanes 1, 2, 4 and 7) or 39 °C (lanes 3, 5, 6 and 8) and infected with RSS-2 wild-type (lanes 2 and 3), A2 wild-type (lanes 4 and 5) or mutant tsA2 (lanes 7 and 8). Lanes 1 and 6 uninfected cells. (b) Immunoprecipitates of PEG precipitates of released virus from the supernatants of the same cultures. Lanes 1, 2, 5 and 7, 33 °C; lanes 3, 4, 6 and 8, 39 °C. Lanes 1 and 4, uninfected cells; lanes 2 and 3, tsA2; lanes 5 and 6, A2; lanes 7 and 8, RSS-2. (c) Lysates of [3H]glucosamine-labelled cells infected with RSS-2 (lanes 1 and 2), or A2 (lanes 3 and 4) or mutant tsA2 (lanes 5 and 6) and immunoprecipitated with anti-G MAb III12a. Lanes 1, 3 and 5, 33 °C; lanes 2, 4 and 6, 39 °C. Lanes M, Mr protein markers.

tsA2- and wild-type-infected cells. The G protein deficiency, therefore, did not appear to be a consequence of variation in the time of radiolabelling in relation to post-translational processing.

(ii) Kinetics of glycoprotein synthesis in tsA2-infected cells

Pulse-chase experiments using [35S]methionine as the radiolabel revealed a marked difference in the time and extent of cleavage of the tsA2 F0 precursor to its subunits F1 and F2 (Fig. 2a). Cleavage was not detectable earlier than 20 to 30 min after pulse-labelling, and even after its onset it proceeded slowly, with the bulk of the F0 precursor remaining uncleaved after 2 h of chase. However, this reduced rate of cleavage relative to the wild-type pattern (Fig. 2b) occurred at both the permissive and restrictive temperatures, suggesting that it is not a temperature-related phenomenon.

The kinetics of G protein synthesis was followed in tsA2-infected cells by pulse-labelling with [3H]leucine and immunoprecipitation with anti-G protein MAb a793 (kindly donated by C. Örvell; Örvel et al., 1987) after varying chase periods (Fig. 2c). At 33 °C the major G protein precursor was detected throughout the duration of the 2 h chase period; the mature G protein appeared 30 min after the pulse and increased with time thereafter. This is consistent with previous observations (Gruber & Levine, 1985a, b; Lambert et al., 1988). The major G protein precursor was also labelled efficiently at 39 °C, indicating that G protein synthesis up to this point was unaffected by the restrictive temperature. However, little or no transition to the mature fully glycosylated G protein was detected, at least during the first 90 min after pulse-labelling. By contrast, a similar experiment with wild-type virus showed that the G protein precursor was chased into the mature G protein form as early as 30 min after the pulse at both permissive and restrictive temperature (not shown).

Taken together these results indicate that the ts phenotype of mutant tsA2 may be due to a failure of G protein maturation at 39 °C. Stable revertants of mutant tsA2 could not be obtained, possibly as a consequence of the complexity of the phenotype of this mutant (i.e. the delayed F0 cleavage).

Evidence suggesting that an M protein defect determines the ts phenotype of mutant tsN1 (complementation group D)

Mutant tsN1 belongs to the collection of mutants isolated by Faulkner et al. (1976) from the RSN-2 (subgroup B) wild-type strain of RS virus. Mutant tsN1 is the solitary representative of complementation group D (Gimenez & Pringle, 1978).
(a) Cells infected with mutant tsN1 (lanes 3 and 6) or wild-type virus (strain RSN-2) (lanes 2 and 5), or mock-infected (lanes 1 and 4) cells at 33 °C (lanes 1 to 3) or 39 °C (lanes 4 to 6), were radiolabelled with [35S]methionine and immunoprecipitated with polyclonal anti-RS virus serum. (b) Immunoprecipitates of cells infected with RSN-2 (lanes 1 and 2), mutant tsN1 (lanes 3 and 4), or a non-ts revertant of tsN1 (lanes 5 and 6), at 33 °C (lanes 1, 3 and 5) and 39 °C (lanes 2, 4 and 6).

(i) Viral polypeptide synthesis at 33 °C and 39 °C in cells infected with mutant tsN1

The P protein of mutant tsN1 migrated more slowly than the RSN-2 wild-type P protein at 33 °C and 39 °C as reported previously (Pringle et al., 1981). In addition the M protein band was absent from the tsN1 profile at 39 °C (Fig. 3a). Several spontaneous non-ts revertants were isolated and examined in a similar fashion to establish which of these two phenotypic properties was associated with the temperature sensitivity of tsN1. The mobility of the P polypeptide of the non-ts revertants was indistinguishable from that of the P polypeptide of the parental tsN1 mutant, and limited enzymic cleavage at acidic residues with S. aureus V8 protease revealed no differences, whereas the corresponding fragments in the wild-type profile migrated faster, similarly to the undigested polypeptides (data not shown). The M polypeptide band, on the other hand, was restored to the revertant profiles at 39 °C (Fig. 3b), confirming that the ts phenotype was associated with absence of the M polypeptide and not with the atypical mobility of the P polypeptide.

Interestingly, the M polypeptides of tsN1 and one non-ts revertant clone translated in vitro migrated slightly, but reproducibly, more slowly than the wild-type polypeptide (Fig. 4a). The same differences could also be detected in vivo (Fig. 4b) and were more noticeable if exposure times were reduced (Fig. 4c). The M proteins of two other non-ts revertant clones isolated independently also exhibited the slightly slower mobility of that of the tsN1 parent, suggesting that all three non-ts clones were probably pseudorevertants.
(ii) Post-translational M protein instability at 39°C in tsN1-infected cells

The kinetics of M protein synthesis were monitored by pulse-chase experiments. In tsN1-infected cells the M protein synthesized during a 20 min pulse at 39°C disappeared from the soluble cytoplasmic fraction within the first 20 min of the subsequent 2 h chase period at 39°C (Fig. 5a). By contrast, the wild-type (Fig. 5b) and revertant profiles (not shown) were identical at both temperatures.

Pulse-labelling at 33°C followed by shift-up to 39°C during the chase period showed that the M protein synthesized under permissive conditions had mostly disappeared by 10 min after shift-up, and was completely absent by 25 min (Fig. 6a). In a similar experiment with one of the revertant clones (Fig. 6b), the M protein remained in the infected cell cytoplasm throughout the 3 h chase period (although some reduction in the intensity of the revertant M protein band was eventually detected at 39°C). These experiments suggest that the tsN1 M protein becomes metabolically unstable at 39°C soon after its synthesis. In the absence of any major post-translational M protein modifications (Cash et al., 1979), the rapid temperature-induced instability implies that the tsN1 M protein may be inherently thermolabile, perhaps due to a conformational change.

Discussion

The association of a G protein deficiency with mutant tsA2 substantiates the hypothesis of Belshe et al. (1977) that mutant tsA2 is defective in adsorption and/or penetration, because it is now clear that the G protein is the attachment protein (Levine et al., 1987). The G protein deficiency involves the mature G protein only because synthesis of the major G protein precursor species p50 was observed at both temperatures. This precursor contains endoglycosidase H-resistant N-linked carbohydrates (Wertz et al., 1989), indicating that it is present in the medial Golgi compartment (Dunphy & Rothman, 1985). Since core O-glycosylation takes place in the cis-Golgi cisternae (Roth, 1984), the endoglycosidase H resistance implies that some O-linked carbohydrates have been added. Addition of O-linked N-acetylgalactosamine residues and modification of N-linked oligosaccharides may account for the difference in mobility of the p50 and p45 forms, because the latter probably represents the cotranslationally glycosylated G protein species in the rough endoplasmic reticulum (Lambert, 1988; Wertz et al., 1989). The presence of p50 in tsA2-infected cells at 39°C indicates that the delay in its maturation may be a consequence of defective transport from the medial to the trans-Golgi cisternae.
Defective core glycosylation per se is unlikely to be responsible for the tsA2 phenotype because unglycosylated wild-type G protein is expressed at the cell surface (Satake et al., 1985; Wertz et al., 1989).

Little is known about the structural features required for vectorial transport of class II (i.e. N-terminally anchored) glycoproteins. C-terminal truncation of the G protein of RS virus indicates that the conformation of the distal region of the ectodomain has little effect on transport (Vijaya et al., 1988; Olmsted et al., 1989; Garcia-Barreno et al., 1990). The absence of intermediates between p50 and the mature G protein (similar to those observed between the p45 and p50 stages), and the identification of p50 as a stable precursor and its late processing (Fernie et al., 1985; Routledge et al., 1986; Walsh et al., 1987; Wertz et al., 1989) together suggest that there may be a pause in G protein maturation at the p50 stage and that subsequent processing proceeds rapidly.

This pause could account for the non-quantitative conversion of p50 into the mature G protein in the pulse-chase experiments described in this and previous studies (Routledge et al., 1986), and may be due to conformational rearrangements. Since processing of core O-linked G protein oligosaccharides appears to occur in a predefined sequence (Wertz et al., 1989), the putative rearrangement of the p50 precursor may reflect transi-
phenotype could thus be interpreted as a disturbance of the presumptive conformational rearrangement of the p50 precursor prior to further processing. The non-ts cleavage defect of the tsA2 F₀ precursor which we have described could account for the non-syncytial phenotype of mutant.

The absence of obvious M protein breakdown products could be due to their small size or failure to react with antibody. Alternatively, the tsN1 M protein may be absent from the soluble fraction of infected cells due to aggregation. Both phenomena have been described for tsM protein mutants of vesicular stomatitis virus (VSV) (Knipe et al., 1977; Ono et al., 1987). The fact that the three revertants examined possess the slightly retarded M protein mobility of tsN1 and the high frequency of isolation of non-ts clones (approximately 10⁻⁴) suggest pseudoreversion, similarly to M protein ts mutants of Newcastle disease virus (NDV) (Peeples & Bratt, 1984) and VSV (Morita et al., 1987). Likewise, the difference in electrophoretic mobility between the wild-type and tsN1 M proteins is a phenomenon shared by tsM protein mutants of NDV (Peeples & Bratt, 1984) and VSV (Knipe et al., 1977; Kennedy-Morrow & Lesnaw, 1984; Pal et al., 1985).

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References


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